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Effects of a low protein diet on calcium metabolism in growing Cashmere goats

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Abstract

The aim of the present study was to determine the effects of a low-protein diet on calcium (Ca) homeostasis and the gene expression related to Ca absorption in the gastrointestinal tract and kidney in growing Cashmere goats. A total of 30 cashmere goats (approximately 4 months old and 15.51±2.3 kg live weight) were divided into a control (10.5%), low (7.5%), or high (13.5%) protein group, with 10 replicates in each group. The experiment was composed of a 15-d adaptation and a 42-d collection period. The low protein group had lower contents of urea, Ca, calcitriol, and insulin-like growth factor-1 (IGF-1) in the plasma and urea and Ca in urine, with substantially higher contents of parathyroid hormone in the plasma and creatinine in the plasma and urine at 21 d and 42 d. The mRNA expression of intestinal vitamin D-dependent 9 ku calcium binding protein (*CaBP-D9k*), plasma membrane calcium ATPase 1b, transient receptor potential vanilloid channel type 6 (*TRPV6*), and the protein expression of intestinal *CaBP-D9k* and *TRPV6* in the low protein group were decreased, whereas the mRNA and protein expression of the vitamin D receptor were not affected. Goats fed a reduced-protein diet showed substantially higher amounts of renal water channel aquaporin 1 (*AQP1*) mRNA in cortical tissue, and the expression of *AQP2* mRNA and protein was elevated in the outer medulla. In conclusion, changing dietary N intake affected Ca metabolism in growing Cashmere goats.

Keywords: calcium absorption, Cashmere goat, low protein diet

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Introduction

For single-stomached animals and humans, protein metabolism is closely associated with homeostatic systems; for example, changing the amount of dietary protein affects calcium (Ca) homeostasis. When fed low protein diets, single-stomached animals and humans demonstrate a decrease in intestinal Ca absorption (Kerstetter *et al.*, 2003; Gaffney-Stomberg *et al.*, 2010), resulting in a decline in plasma calcitriol (1,25-dihydroxyvitamin D_3) concentrations. In addition, it was observed that there was a sharp increase in serum parathyroid hormone (PTH) concentration in humans (Dubois-Ferriere *et al.*, 2011), which probably plays a highly negative role in Ca homeostasis and health, especially bone and kidney functions. As a result, an interaction between protein and Ca metabolism has been explored in monogastric animals and humans (Conigrave *et al.*, 2004).

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Dietary protein is essential for animal growth and health, and it is a vital source of synthetic osteoprotein amino acids (Caroli *et al.*, 2011; Bihuniak & Insogna, 2015). However, resource shortages and nitrogen (N) emissions are the main problems in protein feed application (Machmüller *et al.*, 2006; Wang *et al.*, 2018). In ruminants, feeding low-protein diets is desirable to reduce feed costs and N excretion of the animals and thereby decrease the output of N into the environment. In contrast to single-stomached animals, ruminants are capable of effectively recycling N by rumino-hepatic circulation. With the reduction of dietary N, there is a stronger capacity for renal tubular absorption of urea and ruminal urea transportation (Muscher *et al.*, 2010; Firmenich *et al.*, 2018). The efficiency of ruminants expressing endogenous recycling mechanisms to save N demonstrates that these recycling mechanisms play a role in protecting ruminants from N depletion when dietary N is restricted. An effective rate of protein synthesis by rumen microorganisms is guaranteed by the adaptive responses, and the amino acid source for the host originates from the microbial protein, especially when dietary N is deficient. Accordingly, coping with a reduction in N intake is a unique capability of ruminants. In contrast, monogastric animals and humans cannot use endogenous N sources when consuming a low-protein diet. Previous studies in mature goats have demonstrated that severe mineral homeostasis and bone changes occur on a reduced protein diet (Elfers *et al.*, 2016; Wilkens *et al.*, 2020). However, the effect of reduced dietary protein on Ca homeostasis in young goats, especially Inner Mongolian Cashmere goats, which are one of the best Cashmere goat breeds worldwide, is not yet fully understood.

As a result, it was assumed that the potential interaction between dietary interventions of low protein and Ca homeostasis, as observed in animals with a single stomach, could occur in ruminants. Using growing Inner Mongolian Cashmere goats as experimental animals, the objectives of this study were to determine the effects of a low-protein diet on Ca metabolism and gene expression connected with Ca absorption in the gastrointestinal tract and kidney of growing Cashmere goats.

Materials and Methods

To minimize goat suffering, goats were euthanized under halothane anaesthesia according to the International Guiding Principles for Biomedical Research Involving Animals. The Animal Ethics Committee of the Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences (Hohhot, China), which is responsible for Animal Care and Use in the Inner Mongolia Autonomous Region of China, approved the experimental protocols used in this study (approval number IMAAAHS#1215000046002373XP).

Thirty 4-month-old Albas white Cashmere goats were randomly divided into a control (10.5%), low protein group (LP, 7.5%), or high protein group (HP, 13.5%), with ten animals in each group. Each dietary regimen was maintained for a 15-day adaptation and a 42-day collection period.

Standard methods according to the Association of Official Analytical Chemists (2000) were used for the determination of dry matter (DM, Method No. 930.15), crude protein (Method No. 976.05), Ca, and phosphorous (Method No. 935.13). Acid detergent fibre (ADF) and neutral detergent fibre (NDF) contents were determined according to the basic procedure of Van Soest *et al*. (1991) using an Ankom 220 Fibre Analyser (Ankom Co., USA), and the values were expressed exclusive of residual ash. Heat stable amylase was used in the NDF determination. The three diets were isoenergetic, containing approximately 9.70 MJ metabolizable energy/kg DM. The chemical compositions of the diet are shown in Table 1. The goats were fed three times per day and had free access to water. The dry matter intake (DMI) of each group in the control, low, and high protein groups was 0.601, 0.693, and 0.657 kg/d, respectively.

Table 1 Composition and nutrient levels of experimental diets (DM basis)

Composition	LP	Control	HP
Guinea Grass	500.0	500.0	500.0
Soybean meal	12.5	80.0	162.0
Corn	425.0	307.5	233.0
Wheat bran	40.0	80.0	73.0
Maize germ meal	10.0	20.0	20.0
Premix[1]	5.0	5.0	5.-0
$CaHPO_4$	2.0	2.0	1.5
Limestone	0.5	0.5	0.5
Salt	5.0	5.0	5.0
Total	1000.0	1000.0	1000.0
Nutrient levels (g/kg DM)[2]			
Metabolizable energy （MJ/kg DM）	9.7	9.7	9.7
Dry matter	928.9	932.4	936.5
Crude protein	75.9	105.5	135.1
Calcium	4.2	4.3	4.3
Phosphorous	2.4	3.1	3.3
aNDFom (g)[3]	558.3	590.9	605.7
Non-fibrous carbohydrates (g)	549.7	471.8	415.7
Non-fibrous carbohydrates: Neutral detergent fibre	0.9	0.8	0.7

[1]Supplemental trace elements and vitamins per kilogram of basic diet: Fe （$FeSO_4 \cdot 7H_2O$） 900–1200mg; Cu （$CuSO_4 \cdot 5H_2O$） 150–180mg; Zn （$ZnSO_4 \cdot 7H_2O$） 900–1200mg; Co （$CoCl_2 \cdot 6H_2O$） 2–4mg; VA >50000IU; VD3 10000–15000 IU

[2]Determined from the laboratory analyses

[3]aNDFom (ash-free neutral detergent fibre organic matter) assayed with a heat stable amylase and expressed exclusive of residual ash

Blood samples (5 mL) were taken prior to feeding in the morning on Days 21 and 42 of the sampling period by venupuncture from the vena jugularis using syringes with EDTA covers. Blood plasma was centrifuged at 1200 × *g* for 25 min and frozen at −20 °C until it was analysed. Urine samples were taken by aspiration from the bladder during slaughter.

According to the manufacturer's instructions, a commercial colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to measure the contents of Ca, albumin (Alb), PTH, creatinine, and growth hormone (GH) in plasma. A spectrophotometer (UN2CO-WFT2100, Shanghai, China) at different wavelengths was utilized to measure the absorbance. Standard spectrometric techniques (Sarkar and Chauhan, 1967) were adopted to colorimetrically measure the concentration of Ca in plasma. The inter-assay coefficient of variation (CV%) of Ca was 4.4%, and the intra-assay CV was 2.4%. A normative dye binding technique with bromocresol green was used to detect the concentration of plasma Alb to obtain an inter-assay CV of Alb of 2.3%, and the intra-assay CV was 5.34%. A commercial Coomassie blue protein assay (Bio-Rad, Munich, Germany) was conducted to measure the total protein concentration using bovine plasma gamma globulin as a standard protein to obtain an inter-assay CV of 1.2% and an intra-assay CV of 1.0%. A commercial kit (R-Biopharm, Darmstadt, Germany) was utilized to measure the concentrations of plasma and urine urea to obtain an inter-assay CV of urea of 2.7%, and an intra-assay CV of 1.0%.

The concentrations of insulin-like growth factor-1 (IGF-1) and calcidiol were determined using enzyme-linked immunosorbent assay (ELISA) with commercial kits based on the respective manufacturer's protocol (Immundiagnostik AG, Bensheim, Germany). In brief, standard samples were

diluted continuously. Samples were added to testing sample wells. After 37 °C incubation for 30 min, the plate was rinsed, and HRP-conjugated reagent was added. Blood calcitriol levels were measured by radioimmunoassay (Alexandra *et al*., 2012).

The goats were slaughtered on day 42, and the gastrointestinal tract and kidney tissue were obtained for RNA isolation. TRIzol solution (TaKaRa, Inc. Dalian, China), in accordance with the manufacturer's instructions, was used to extract the total RNA, and the integrity and purity were evaluated using agarose gel electrophoresis (2%) and a microplate reader. The final 20 µL volume, including 2 µL of 5 × PrimeScript Buffer, 0.5 µL of PrimeScript RT Enzyme Mix I, 0.5 µL Oligo dT Primer (50 µM), 0.5 µL Random-6-mers (100 µM), and 6.5 µL RNA reverse transcriptase, was used to conduct reverse transcription (RT) reactions. The RT reaction parameters were as follows: RT for 15 min at 37 °C and RT inactivation for 5 s at 85 °C. The RT products (cDNA) were stored at -20 °C for the quantitative PCR assay.

The primers used in this study are shown in Table 2. Relative levels of gene expression were quantified by using the SYBR® Prime Script™ RT–PCR Kit (TaKaRa, Inc. Dalian, China) according to the manufacturer's instructions. In brief, the PCR system (20 µL) contained 10 µL of SYBR® Premix Ex Taq™ (2 ×), 7.2 µL of dH_2O, 0.4 µL (10 µM) of forwards and reverse primers, and 2 µL of cDNA template.

Table 2 Primer sequences used for Reverse transcription polymerase chain reaction (RT-PCR)[1]

Genes	Primer sequences (5'-3')	Length	GenBank no.
AQP1	F:GACACCTGCTGGCGATAGAC R:TGGTCCTGGAAATTGTGCGT	91bp	XM_018047113.1
AQP2	F:GGCCGTTACTGTGGAGCTTT R:TGTAGTGGATCCCAAGGAGGT	149bp	XM_018047814.1
CaSR	F:AGAGGACGGCTCCATAGTGT R:ACAGAGGGGCTCCCTTGAG	125bp	XM_018059989.1
VDR	F:CCAGTTCGCAAGGATGAGG R:CTGGTTGGCTCCGTTGTGT	131bp	XM_018047872.1
TRPV6	F:TCACCTACGCTGCCTTCGC R:ATCCCGCTCGTGGGCTACAC	101bp	XM_018046847.1
NCX1	F:AGTGCGCCGTTTTTCCTACT R:CCAACTGTCCCAACCTACTGG	84bp	XM_018055160.1
CaBP-D9K	F:AGCACCCTCGATGAGCTTTT R:TGGCGACATTGATGGTTTCG	185bp	XM_005701057.2
PMCA1b	F:AGCAGTTATGTGGGGACGAAA R:GCCCGTGAAAGCAACAATC	100bp	XM_018047613
Cldn-2	F:AGCTACAGCCAGCAGACAAG R:TGCTGGCACCAACATAGGAG	177bp	XM_005700206.3
Cldn-12	F:GTGTGGGCGAGTGAAAATGC R:ACTGCCAGTCAGATCTTTGAGG	133bp	XM_005678898.3
β-actin	F:GGCGTGAACCACGAGAAGTA R:GGCGTGGACAGTGGTCATAA	158bp	NM_0010097

[1]*AQP* = water channel aquaporin. *CaSR* = Ca sensing receptor. *VDR* = the vitamin D receptor.*TRPV6* = transient receptor potential vanilloid channel type 6.*NCX1* = Na^+/Ca^{2+}-exchanger 1. *CaBP-D9k* = vitamin-D dependent 9 ku calcium binding protein. *PMCA1b* = plasma membrane calcium ATPase 1b. *Cldn* = claudin

A Bio-Rad iCycler IQ5 detector (Perkin Elmer-Applied Biosystems, Foster City, CA) was utilized to conduct real-time PCR according to the following procedure. The first step was a 30 s initial denaturing step at 95 °C. The second step was 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C and 20 s extension at 72 °C. The $2^{-\Delta\Delta CT}$ method was adopted to calculate the quantitative data of real-time PCR (Livak and Schmittgen 2001). After being separated by electrophoresis on a 2% agarose gel, the amplified products were detected under ultraviolet light.

The protein expression of the small intestinal vitamin D-dependent 9 ku calcium binding protein (CaBP-D9k), transient receptor potential vanilloid receptor 6 (TRPV6), vitamin D receptor (VDR), claudin-2 (Cldn-2), and renal aquaporin 1 (AQP1) and AQP2 were detected using western blot analysis. The tissue was lysed in modified Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Beijing, China). The lysates (50 µg of protein per lane) were fractionated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluorine membranes. Later, the blocked membranes were incubated with primary antibodies, followed by peroxidase-conjugated secondary antibodies, and Enhancer Chemiluminescence (Beyotime, Beijing, China) was utilized to visualize protein bands using autoradiography Image Quant software (GE Healthcare) to scan and qualify the autoradiographs.

Data were evaluated using analysis of variance and Duncan's multiple range test of SAS software (SAS Version 9.0, SAS Institute, Cary, NC). A level of $P < 0.05$ was used as the criterion for statistical significance, while a level of 0.10 was taken to indicate a statistical trend.

Results and Discussion

Table 3 shows the effect of the low protein diet on blood parameters in growing Cashmere goats. In comparison with the high protein group, the low protein group had lower contents of urea, Ca, calcitriol, and IGF-1 in plasma and urea and Ca in urine ($P < 0.05$), with higher contents of PTH in plasma and creatinine in plasma and urine ($P < 0.05$) at 21 d and 42 d.

Ca homeostasis is crucial for the normal function of the organism. Despite effective N recycling pathways, dietary protein restriction affected Ca homeostasis in goats in our study. Similarly, it was reported that providing a low-protein diet after 6 weeks substantially decreased the blood Ca concentration in male White Saanen goats (Muscher & Huber, 2010). Moreover, a previous study showed no change in plasma Ca in female Xiongdong black goats fed a low protein diet, which was contradictory to our results (Mi *et al.*, 2022). This difference could be ascribed to inconsistencies in the source of protein and experimental methodology (Roughead, 2003). Meanwhile, different animal species may have various adaptations to the change in dietary protein levels. Additionally, a substantial reduction in plasma total Ca and urea levels was observed in our study. Muscher & Huber (2010) showed that this interaction of N and Ca metabolism was also indicated by a linear relationship between plasma urea levels, which are used as an indirect parameter for N intake and plasma Ca concentration. In our study, a remarkable decrease in the concentration of plasma urea was caused by the decrease in dietary N. In previous studies, a renal decrease in the urea excretion rate was obtained as a result of renal mechanisms conserving urea during N deprivation, whereas the glomerular filtration rate fell by approximately 60% with the decrease in N intake (Wilkens & Muscher, 2020). Reduced IGF-1 concentrations can result in a reduction in glomerular filtration rate (GFR), which could modulate GFR in goats (Elfers *et al.*, 2014). Muscher *et al.* (2011) revealed that the decrease in GFR was denoted by a rise in the concentration of plasma creatinine in goats fed a decreased N diet. However, as demonstrated by the simultaneous rise in the concentrations of urinary creatinine, it seems that a higher muscle turnover causes an increasing release of creatinine from muscle. A higher turnover of muscle protein would provide urea synthesis in the N-reduced goats with more N to increase N supply to ruminal microorganisms. Undoubtedly, GFR is related to the concentration of plasma calcitriol in goats (Herm *et al.*, 2015).

There is another reason that the IGF-1 concentration impacts the activity of renal1-a hydroxylase. This enzyme converts calcidiol to calcitriol (Muscher & Huber, 2010). Furthermore, a decrease in calcitriol concentrations was shown in goats fed a low-protein, decreased-N diet in our study, as well as in rats fed a reduced N diet (Orwoll *et al.,* 1992).

As shown in Tables 4 and 5 and Figure 1, the low protein group had lower mRNA expression of *Cldn-2*, *Cldn-12*, *CaBP-D9k*, plasma membrane calcium ATPase (*PMCA1b*), and *TRPV6* and protein expression of Cldn-2, CaBP-D9k and PMCA1b (P <0.05) in comparison with the high protein group. However, there was no prominent discrepancy between the low protein group and the control (P >0.10).

Table 3 The biochemical indices in blood and urine samples from Cashmere goats on high- and low-protein diets

Position	Item[1]	LP	Control	HP	SEM[2]	P-value
Blood	**21 d**					
	Urea (mmol/L)	8.16^{b}	9.70^{b}	12.34^{a}	0.56	0.007
	Creatinine (μmol/L)	33.25^{a}	25.00^{b}	20.00^{b}	1.59	0.001
	TP (g/L)	32.13	35.98	38.05	4.37	0.640
	Alb (g/L)	16.00	17.00	19.88	2.84	0.620
	Ca (mmol/L)	1.08^{b}	1.55^{a}	1.80^{a}	0.12	0.012
	Calcitriol (ng/L)	59.78^{b}	69.26^{b}	90.37^{a}	3.29	0.001
	Calcidiol (mmol/L)	1.37	1.65	1.83	0.19	0.283
	IGF-1 (ng/mL)	33.94^{b}	49.29^{ab}	60.35^{a}	5.06	0.015
	GH (ng/mL)	0.86	0.41	0.58	0.24	0.450
	PTH (ng/L)	28.41^{a}	26.08^{a}	21.68^{b}	1.26	0.013
	42d					
	Urea (mmol/L)	7.61^{c}	9.86^{b}	12.09^{a}	0.64	0.006
	Creatinine (μmol/L)	34.50^{a}	21.75^{b}	19.50^{b}	2.28	0.003
	TP (g/L)	36.60	38.43	39.60	6.30	0.940
	Alb (g/L)	19.13	19.75	21.10	3.06	0.890
	Ca (mmol/L)	1.21^{c}	1.63^{b}	1.98^{a}	0.08	0.000
	Calcitriol (ng/L)	69.59^{a}	77.41^{ab}	92.44^{a}	4.39	0.032
	Calcidiol (mmol/L)	1.25	1.60	1.69	0.25	0.435
	IGF-1 (ng/mL)	32.84^{c}	39.73^{b}	48.78^{a}	1.72	0.001
	GH (ng/mL)	0.69	0.41	0.56	0.17	0.550
	PTH (ng/L)	27.67^{a}	23.27^{b}	20.95^{c}	0.57	<0.0001
Urine	Ca (mmol/L)	0.49^{b}	1.45^{a}	1.75^{a}	0.11	0.007
	Urea (mmol/L)	27.10^{b}	52.80^{a}	62.97^{a}	3.57	0.001
	Creatinine (μmol/L)	711.33^{a}	546.67^{b}	240.67^{c}	13.64	<0.0001

[1]TP: total protein; Alb: albumin; Ca: calcium; IGF-1: insulin-like growth factor-1; GH: growth hormone; PTH: parathyroid hormone; LP: low protein, 7.5% CP; HP: high protein, 13.5% CP; Control, 10.5% CP

[2]SEM: standard error of the mean

[a-c] Means in the same row not followed by the same letter differ significantly (P <0.05), whereas the differences were considered to be a statistical trend at 0.05 <P <0.10

As shown in Tables 6 and 7 and Figure 2, the mRNA expression of renal cortex *AQP1* as well as the mRNA and protein expression of renal medulla *AQP2* were upregulated in the low protein group (P <0.05). The protein levels of diets had no effect on the mRNA expression of *CaSR* in the cortex and medulla of the kidney (P >0.05).

Table 4 The mRNA expression of Ca uptake-related genes in the small intestine of Cashmere goats fed a high- and low-protein diet

Genes[1]	LP	Control	HP	SEM[2]	P-value
CaBP-D9k	0.651[b]	1.036[b]	2.154[a]	0.201	0.0001
PMCA1b	0.786[b]	1.025[b]	1.832[a]	0.106	0.001
TRPV6	0.724[b]	1.029[b]	1.820[a]	0.127	0.002
NCX1	1.371	1.071	1.567	0.166	0.183
VDR	0.958	1.068	1.256	0.112	0.354
Clnd2	0.866[b]	1.187[b]	1.945[a]	0.677	0.003
Clnd12	0.669[b]	1.004[b]	1.830[a]	0.289	0.015

[1]*CaBP-D9k*: vitamin-D dependent 9 ku calcium binding protein; *PMCA1b*: plasma membrane calcium ATPase 1b; *TRPV6*: transient receptor potential vanilloid channel type 6; *VDR*: the vitamin D receptor; *NCX1*: Na+/Ca2+-exchanger 1; *Cldn*: claudin; LP: low protein, 7.5% CP; HP: high protein, 13.5% CP; Control, 10.5% CP
[2]SEM: standard error of the mean
[a-c] Means in the same row not followed by the same letter differ significantly (P <0.05), whereas the differences were considered to be a statistical trend when 0.05 <P <0.10

Table 5 The protein expression of Ca uptake-related genes in the small intestine of Cashmere goats fed a high- and low-protein diet

Genes[1]	LP	Control	HP	SEM[2]	P-value
CaBP-D9k	0.66[b]	0.71[b]	1.35[a]	0.052	0.000
TRPV6	0.78[b]	0.85[b]	1.24[a]	0.038	0.015
VDR	0.79	0.66	0.56	0.031	0.582
Cldn-2	0.75[b]	1.19[b]	1.79[a]	0.048	0.024

[1] *CaBP-D9k*: vitamin-D dependent 9 ku calcium binding protein; TRPV6: transient receptor potential vanilloid channel type 6; VDR: the vitamin D receptor; Cldn: claudin; LP: low protein, 7.5% CP; HP: high protein, 13.5% CP; Control, 10.5% CP
[2]SEM: standard error of the mean.
[a-c] Means in the same row not followed by the same letter differ significantly (P<0.05), whereas the differences were considered to be a statistical trend when 0.05 <P<0.10.

Table 6 The mRNA expression of Ca uptake-related gene in the kidney of Cashmere goats fed a high- and low-protein diet

Item	Genes[1]	LP	Control	HP	SEM[2]	P-value
cortex	*CaSR*	1.015	1.008	1.272	0.252	0.398
	AQP1	1.896[a]	1.050[b]	0.822[b]	0.135	0.003
	AQP2	1.021	1.100	1.027	0.198	0.952
medulla	*CaSR*	1.024	1.073	1.165	0.232	0.880
	AQP1	1.114	1.131	1.077	0.131	0.957
	AQP2	1.937[a]	1.035[b]	0.880[b]	0.074	0.000

[1] CaSR: Ca sensing receptor; AQP: water channel aquaporin; LP: low protein, 7.5% CP; HP: high protein, 13.5% CP; Control, 10.5% CP
[2] SEM: standard error of the mean
[a-c] Means in the same row not followed by the same letter differ significantly (P <0.05), whereas the differences were considered to be a statistical trend when 0.05 <P <0.10

Table 7 The protein expression of Ca uptake-related gene in kidney medulla of Cashmere goats fed a high- and low-protein diet[1]

Genes[1]	LP	Control	HP	SEM[2]	P-value
AQP1	1.092	0.973	0.961	0.123	0.854
AQP2	1.644[a]	1.345[b]	1.186[b]	0.253	0.008

[1] AQP: water channel aquaporin; (LP: low protein, 7.5% CP; HP: high protein, 13.5% CP; Control, 10.5% CP

[2] SEM: standard error of the mean

[a-c] Means in the same row not followed by the same letter differ significantly (*P* <0.05), whereas the differences were considered to be a statistical trend when 0.05 <P <0.10

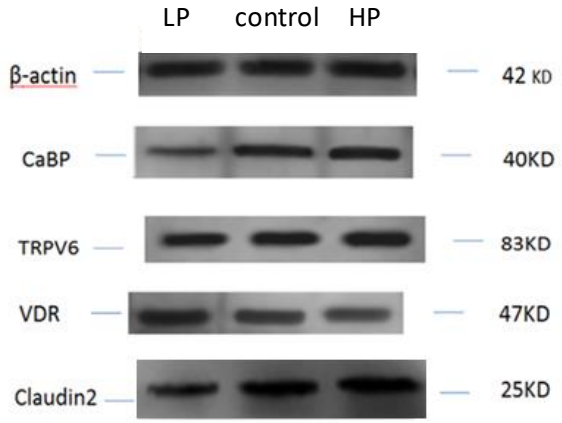

Figure 1 Changes in the protein expression of Ca uptake-related genes in the small intestine of Cashmere goats fed a high- and low-protein diets. The protein expression of the small intestinal *CaBP-D9k, TRPV6, VDR, Cldn-2* were detected using western blot analysis (LP: low protein, 7.5% CP; HP: high protein, 13.5% CP; Control, 10.5% CP)

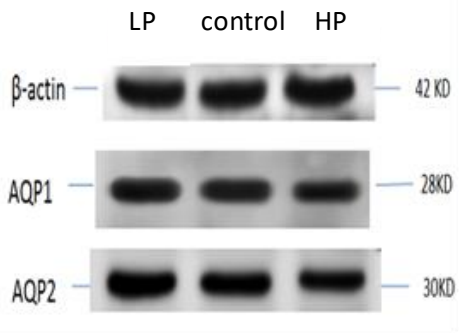

Figure 2 Changes of the protein expression of Ca-uptake-related genes in the kidney of Cashmere goats fed a high- and low-protein diet. The protein expression of renal *AQP1* and *AQP2* were detected using western blot analysis (LP: low protein, 7.5% CP; HP: high protein, 13.5% CP; Control, 10.5% CP)

By coordinating the availability of Ca in the blood at the level of the bones, intestine, and kidneys, the Ca homeostasis of ruminants under normal physiological conditions is regulated by the classical ciotropic hormones, PTH, calcitriol, and calcitonin (CT). Calcitriol, also known as the active form of vitamin D ($1,25\text{-}(OH)_2D_3$), increases Ca concentrations in blood by increasing both intestinal Ca absorption and renal Ca reabsorption (Elfers *et al.*, 2015). In the intestine, the active transcellular pathway is the major means by which calcitriol regulates Ca absorption. Intestinal transcellular transportation includes Ca entry through specific Ca channels, such as TRPV5, in brush border membranes, and then intracellular transport is mediated by the calcium-binding protein CaBP-D9k. Ca is passively transported to the bloodstream by PMCA1b or the Na^+/Ca^{2+}-exchanger (NCX1) (Hoenderop *et al.*, 2005). These steps are regulated by the vitamin D receptor (VDR) (Song *et al.,* 2003; Kim *et al.,* 2009). Lieben *et al.* (2011) reported that calcitriol increases the expression of Ca transporters (i.e., calbindin-D9k, calbindin-D28k, PMCA1b, TRPV5, and TRPV6) in the intestine and

kidney. Calcitriol also increases the efficiency of passive paracellular transport of Ca by increasing the expression of Cldn-2 and Cldn-12 in the gut (Lieben *et al.,* 2011; Elfers *et al.*, 2016), which is still not understood completely. In our study, the protein expression of the Ca channel, *TRPV6,* and the Ca transporter, *CaBP-D9k*, as well as the mRNA expression of *CaBP-D9k*, *PMCA1b,* and *TRPV6* were decreased in the goats in the 7.5% CP group in comparison with animals in the 13.5% CP group, while there was no effect on the protein and mRNA expression of the *VDR* in either tissue. These results are consistent with previous studies showing that a protein-restricted diet decreases the protein expression of TRPV6 but does not affect the protein expression of VDR in the jejunum of growing White Saanen goats (Muscher *et al.*, 2012). The reduction in TRPV6 and CaBP-D9k protein expression could be attributed to the low concentration of plasma calcitriol in the 7.5% CP group, presuming that these processes were calcitriol dependent, as in animals with a single stomach (Firmenich *et al.*, 2018).

Goats in the present study fed the low protein diet showed a reduction in plasma urea concentration and a concomitant reduction in renal urea excretion. In a previous study, an N-decreased diet in young goats resulted in an upregulation of urea transporter, UT-A1, mRNA expression in the renal medulla (Starke *et al.*, 2012), presuming that when N was scarce, it is significant in conserving urea. A urinary-concentrating defect in rats and other monogastric animals is caused by changes in urea metabolism by low protein diets, which presumably is related to changes in the expression of renal water channels called AQP (Elfers *et al.*, 2014). Both AQP1 and AQP2 are mainly involved in regulating renal transcellular water flow. In addition to regulating water homeostasis, the kidney is a key organ modulating Ca homeostasis due to CaSR, which is expressed along the whole nephron and senses extracellular Ca levels in urinary filtrate and interstitial fluid (Riccardi & Brown, 2010). The CaSR functions in monogastric animals and is a potential modulator of renal AQP2 expression (Bustamante *et al.*, 2008). In the current study, the alteration in Ca homeostasis resulted in a decrease in dietary protein in young goats, similar to a decrease in calcitriol concentrations. Various studies in monogastric animals have demonstrated inconsistent results related to the effect of calcitriol on CaSR expression (Brown *et al.,* 1996). An N-reduced diet can result in large alterations in urea metabolism and mineral homeostasis in young goats (Muscher *et al.,* 2010; Muscher *et al.*, 2011; Starke *et al.*, 2012). In the current study, a decreased concentration of plasma urea and a rise in renal urea transporters was observed in goats fed an N-decreased diet. The alteration of plasma calcitriol did not modify the expression of CaSR; thus, the involvement of CaSR in renal AQP2 modulation can be eliminated in dietary N reductions in young goats. Elfers (2016) pointed out that renal medullary AQP2 mRNA and protein expression rose at least partly on the basis of increased plasma concentrations of antidiuretic hormone/vasopressin, in goats fed decreased N, which may be related to improved renal urea reabsorption based on the strong capability of small ruminants to offset low intake of dietary N by raising endogenous urea recycling. Nevertheless, antidiuretic hormone/vasopressin was not measured in our experiment; thus, it is necessary to conduct further investigations to test the actual mechanism.

Conclusion

In summary, the present study showed that a low protein diet in young Cashmere goats led to decreased calcitriol and IGF-1 concentrations and increased PTH concentrations. Furthermore, Ca homeostasis was perturbed in goats on the low protein diet, despite N recycling mechanisms. Moreover, a low protein diet upregulated the mRNA expression of AQP1 and the mRNA and protein expression of AQP2, suggesting that goats with insufficient dietary protein may maintain the balance of calcium metabolism through the kidney signalling pathway. In conclusion, an interaction between dietary N and Ca metabolism occurs in young goats as in monogastric animals. The dietary requirements of N for young goats must be investigated further to decrease N pollution, especially with a focus on mineral metabolism.

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Author Contributions

LJ and HZS contributed to the project idea, design, and execution of the study. RGNM contributed to laboratory analyses. SLL and CHZ analysed the data. LJ drafted and wrote the manuscript. DS and CZZ critically reviewed the manuscript. All authors have read and approved the finalized manuscript.

Conflict of Interest Declaration

The authors have no conflicts of interest to declare.

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